Thiophilic Interaction Chromatography of Human Transferrins

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Abstract

Lactoferrin and serum transferrin, the iron-binding proteins, are widely distributed in biological fluids. Transferrins have many biological functions: anti-bacterial, anti-inflammatory, and immunoregulatory. A facile monitoring of the concentration of transferrins in biological fluids may have medical relevance. Thiophilic-interaction chromatography of transferrins may provide an efficient means for their quantitation in serum.

Introduction

Transferrins, lactoferrin and serum transferrin, are a class of iron-binding proteins that are widely distributed in biological fluids. Lactoferrin is an iron-binding protein found in blood, milk, and exocrine secretions, including nasal, pancreatic juice, lachrymal, vaginal, seminal, as well as specific granules of neutrophils (1). Lactoferrin is highly basic and interacts with many acidic molecules (2). The molecular weight of lactoferrin is 80 kDa, and it is composed of 703 amino acids residues (3). It is a potent inhibitor (4) of colony stimulating factor and has a nutritional (5), bacteriostatic (6), and immunological role in the body (7). The main function of lactoferrin is to provide a primary defense against microbial infection, mainly because of its ability to sequester iron, which is necessary for microbial growth. In vitro, lactoferrin has been shown to inhibit several strains of bacteria, including Staphylococcus and E. coli. This anti-bacterial effect is lost when lactoferrin is saturated with iron. Lactoferrin can be given as a nutritional supplement for any adult and can be given specifically to people with iron deficiencies. Lactoferrin may be involved in the development of a variety of neurodegenerative disorders. It has been found in senile plaques and neurofibrillary tangles in Alzheimer's disease patients (8) and in some microglia, astrocytes, and oligodendrocytes (9).

Serum transferrin is the principal iron-binding protein in vertebrates (10). It is a bilobal 80 kDa protein and is composed of 684 amino acids residues (11). The two iron-binding sites are structurally similar and bind iron strongly at physiologic pH, in a process that involves a great deal of tertiary structural rearrangement (12). The rate of release of iron from serum transferrin depends on factors such as pH, concentration and type of ionic species present, and temperature. It transports iron from sites of absorption and heme degradation to sites for storage and utilization. It plays a role in the stimulation of cell growth (13) and is capable of binding iron and maintaining it in a relatively non-reactive state (14), thereby providing an antioxidant defense.

Purification of both transferrins has been accomplished by dye–ligand chromatography (15) and anion-exchange chromatography (16). Their purification by thiophilic interaction chromatography (TIC) is now reported. TIC was introduced by Porath et al. in 1985 (17). It has been shown that TIC is an effective method for studying the binding of proteins containing clusters of aromatic residues such as tryptophan, phenylalanine, and tyrosine (18–23). This study evaluates the application of TIC in the isolation of several proteins, which are recognized as biomarkers for various cancers. Recently, TIC has been exploited in the isolation of prostate-specific antigen (24,25). TIC of amyloid peptides (Alzheimer's) has also been accomplished (26). The isolation of lactoferrin and serum transferrin by TIC is reported in this study.

Experimental

The following components constituted the high-performance liquid chromatography (HPLC) setup used for the TIC: Waters 600 multi-solvent delivery system (Waters Millipore, Milford, MA) with 200 µL pumpheads; Rheodyne Model 7125 syringe loading sample injector (Rheodyne, Rohnert Park, CA) with a 2-mL sample loop; Waters 994 programmable photodiode array detector; Waters 470 fluorescence detector; microflow cell and pH microelectrode from Amersham Pharmacia Biotechnology (Piscataway, NJ); and baseline 810 chromatography work station software from Waters Millipore, installed on a HP vectra PC (HP, Palo Alto, CA).

For TIC of human serum, a step-wise gradient mode proved to be the best method for the analysis of the lactoferrin and serum transferrin levels in serum. The gradient program for serum analysis included the following protocol: 0–15 min, 1M

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Na_2SO_4 (20mM phosphate at pH 7.4); 15–35 min, 0.7M Na_2SO_4 (20mM phosphate at pH 7.4); and 35–60 min, 20mM phosphate buffer. The serum was diluted in 5 mL of phosphate buffer saline buffer. The proteins in the TIC of transferrins were detected using the Waters 994 photodiode detector. The detector monitored absorbances from 200 to 400 nm. The plot of the spectra was acquired using the software from the Waters 470 fluorescence detector. The solvents used for the experiments were 1M Na_2SO_4 (20mM phosphate at pH 7.4) and 20mM phosphate buffer. The column was washed with 10 column volumes (30 mL) of starting solution (1M Na_2SO_4, 20mM phosphate at pH 7.4) before each experiment. The experiments were performed following a salt gradient. The transferrins would bind to the thiophilic gel (Sigma-Aldrich, St. Louis, MO) at a high concentration of lyotropic salt and be released as its level was decreased. All TIC of transferrins were performed with an initial wash: 0–10 min (1M Na_2SO_4, 20mM phosphate at pH 7.4) and then with a 10–60 min linear gradient followed by 20mM phosphate buffer alone.

The serum used in the experiments and samples of human lactoferrin and serum transferrin were purchased from Sigma Aldrich Chemicals. The Fractogel Edward Merck Darmstadt Thiophilic Adsorption (3S) was purchased from EM Separation Science (Gibbstown, NJ). All samples of transferrins were prepared by dissolving 4 mg of commercially purchased protein in 1 mL of 1M Na_2SO_4 (20mM phosphate at pH 7.4). Prior to injection of the sample, the sample was centrifuged. An HR 10/2 column was purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ). The column was designed for use on a fast-performance liquid chromatography (FPLC) system with the appropriate FPLC–HPLC unions (P626 10-32 adapter, Amersham Biosciences, Piscataway, NJ). This column was packed with fractogel EMDTA (3S) 3S thiophilic gel. All experiments were performed at a constant flow rate of 0.5 mL/min at room temperature (20°C). All solvents were degassed and purged using helium gas in accordance with the Waters recommendations.

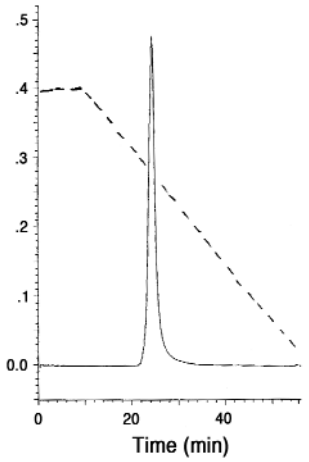

Figure 3. TIC of human serum transferrin (run in a gradient mode) at a flow rate of 0.5 mL/min (0–10 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4, with a linear gradient from 10–60 min).

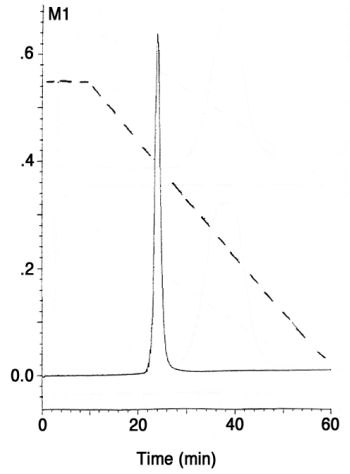

Figure 4. TIC of human lactoferrin on 3S gels (run in a gradient mode) at a flow rate of 0.5 mL/min (0–10 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4 and linear gradient from 10–60 min).

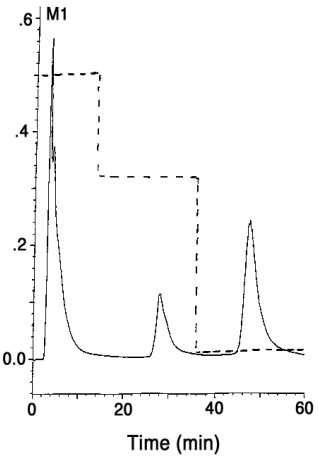

Figure 5. TIC of human serum in a step-wise mode run at a flow rate of 0.5 mL/min (0–15 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4, 15–35 min in 0.7M sodium sulfate with 20mM phosphate buffer and 35–60 min in phosphate buffer alone). The peaks numbers are: human serum albumin, 1; human transferrins, 2; and Igg, 3.

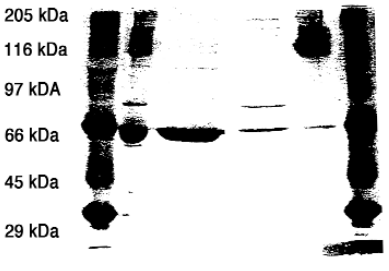

Figure 6. SDS–PAGE of human serum and the three major peaks obtained from T-gel. The six different lanes are: molecular markers, lane 1; human serum, lane 2; peak 1 from serum after the run on T-gel, lane 3; peak 2 from serum after the run on T-gel, lane 4; peak 3 from serum after the run on T-gel, lane 5; and molecular weight markers, lane 6.

Results

Lactoferrin is an iron-binding bilobal protein of the transferrin family. The molecular weight of lactoferrin is 80 kDa, and its crystal structure has been determined by single crystal X-ray diffraction (27). The molecular weight of serum transferrin is 80 kDa, and it is a principal iron-binding protein in vertebrates. It has two binding sites for iron, one in the N and C lobes (28). Figures 1 and 2 (See page 3A) provide a view of their tertiary structures of human lactoferrin and serum transferrin (taken from the Protein databank). One can observe the presence of several aromatic residues on the surfaces of these two transferrins, a fundamental requirement for their affinity towards thiophilic gels (22).

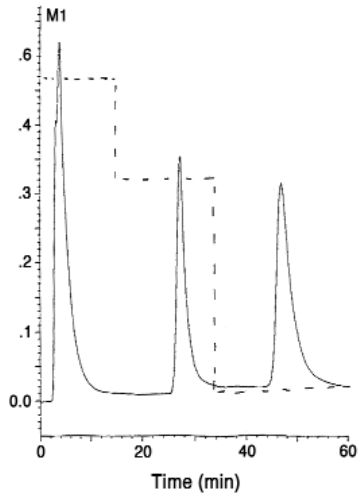

Figure 7. TIC of human serum spiked with serum transferrin on 3S gel at a flow rate of 0.5 mL/min (0–15 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4, 15–35 min in 0.7M sodium sulfate with 20mM phosphate buffer and 35–60 min in phosphate buffer alone).

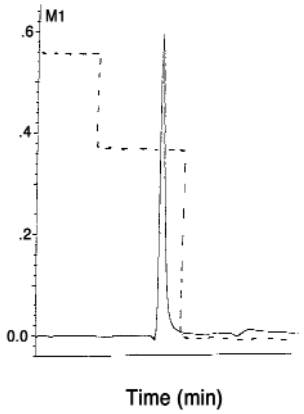

Figure 8. TIC of human transferrin and human lactoferrin on 3S gel at a flow rate of 0.5 mL/min (0–15 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4, 15–35 min in 0.7M sodium sulfate with 20mM phosphate buffer and 35–60 min in phosphate buffer alone). The peak numbers are: human serum albumin, peak 1; human transferrins, peak 2; and Igg, peak 3. Note the binding peak is identical to the second peak from human serum shown in Figure 5.

Figures 3 and 4 demonstrate the chromatography of human serum transferrin and human lactoferrin on a 3S thiophilic gel. Clearly, both human transferrins were retained on the 3S gel, when applied at a high concentration of Na_2SO_4 (1M), They were then recovered in a falling concentration gradient of Na_2SO_4 (1M→0.0M). Both transferrins, in their holo (saturated with iron) and apo forms (no iron), bind to 3S gel.

Many other transferrins and lactoferrins: bovine, rabbit, dog, mouse, rat, and ovotransferrins display an affinity to thiophilic gels, comparable with that of human transferrins (not illustrated).

Having established the elution conditions of both transferrins in a gradient mode (0.7M Na_2SO_4), a sample of human serum was chromatographed on the 3S gel, in a step-wise mode: adsorption at 1M Na_2SO_4 and elution at 0.7M Na_2SO_4, followed by omission of Na_2SO_4 in the elution buffer.

Figure 5 illustrates the distribution of serum proteins: a major fraction of unretained proteins, a smaller fraction eluted at 0.7M Na_2SO_4, and the larger one eluted in the absence of Na_2SO_4 in the eluent. Figure 6 shows the sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) of these three fractions. Remarkably, the second fraction at 0.7M

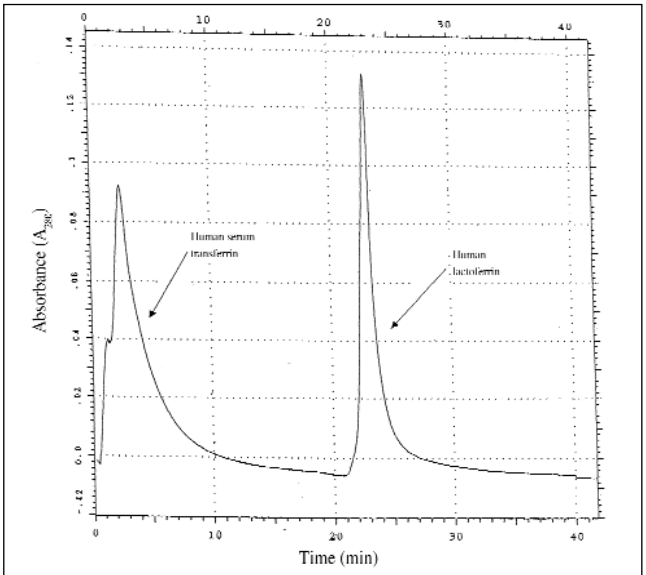

Figure 9. Resolution of peak 2 from human serum into the major fractions of human lactoferrin and human serum transferrin on the mercaptoethylpyridine (MEP) column. (0–15 min in 20mM phosphate buffer at pH 7.4 and 15–40 min in 20mM acetate buffer at pH 4.0).

Figure 10. SDS–PAGE of the two major fractions from the MEP column, which are human serum transferrin and human lactoferrin.

Na_2SO_4 displayed a rather limited number of bands, indicating the usefulness of this chromatographic procedure for the isolation of human transferrins. To confirm this observation, a sample of human serum was supplemented with purified human serum transferrin and lactoferrin (commercial products). Figure 7 illustrates a significant increase in the second fraction, which is expected to contain transferrins resident in the serum. In order to confirm this finding, a mixture of human serum transferrin and lactoferrin was chromatographed in the step-wise elution procedure. As expected, both transferrins were recovered from the second eluted fraction (Figure 8). Both human serum transferrin and lactoferrin display remarkably similar affinity for a thiophilic gel (3S), as illustrated in Figure 8.

In order to resolve them, hydrophobic charge-induction chromatography (HCIC) was used (29). Figure 9 shows that human serum transferrin was not retained, though human lactoferrin was eluted only after the pH of the eluting buffer was diminished from pH 7.4 to 6.0. The SDS–PAGE (Figure 10) illustrates the relatively high purity of the transferrin in the serum (second fraction on 3S gel).

Conclusion

Lactoferrin is an iron-binding protein. It is present in milk, epithelial secretions, and in secondary granules of neutrophils (30). Lactoferrin is a pleiotropic factor with potent antimicrobial and immuno-modulatory activities. Recent studies have demonstrated that it promotes bone growth (31). Serum transferrin, by sequestering iron, provides an anti-oxidation defense. A disturbed iron metabolism can lead to degenerative disorders. When serum transferrin levels are at their physiologically normal levels in the brain, it is likely to protect against the onset and progression of Alzheimer's disease. It has been found that lactoferrin and serum transferrin levels are decreased in the white matter of the brains of Alzheimer's patients (8).

A facile and accurate method of monitoring levels of transferrins in serum (32) could provide a means for monitoring the onset and progression of Alzheimer's disease. Thiophilic interaction chromatography may provide a simple technique for the isolation of both transferrins. HCIC allows for a ready separation of these two transferrins after their initial isolation from serum by TIC. A systematic follow up study of the sera of Alzheimer's patients is underway at present.

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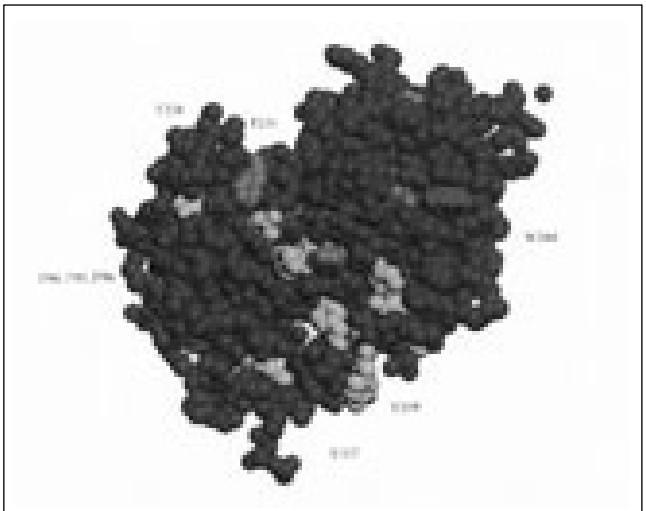

Figure 1. Tertiary structures of serum transferrin at 2.5Å with the aromatic residues Y in yellow, F in magenta, and W in red. All the other residues are in blue. Note the presence of several aromatic residues on the surface of the protein.

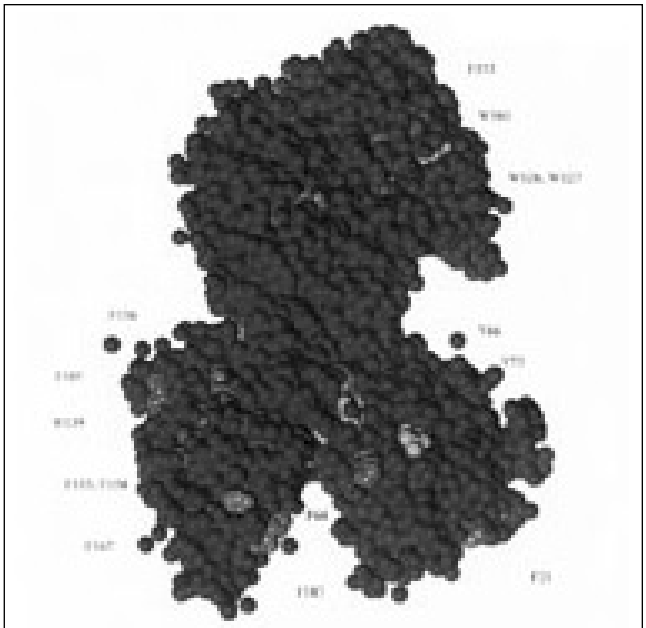

Figure 2. Tertiary structure of human lactoferrin at 2.5Å with the aromatic residues Y in yellow, F in magenta, and W in red. All the other residues are in blue. Note the presence of several aromatic residues on the surface of the protein.